A Cesium Chloride Flotation Technique for the Isolation of Verticillium dahliae Microsclerotia from Soil

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ABSTRACT

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A quantitative technique for the isolation of a low level of microsclerotia of *Verticillium dahliae* from loess soil in Israel is described. Cesium chloride water solution (1:1, w/v) was found suitable for the separation of microsclerotia from soil particles due to its high specific gravity, low viscosity, and low toxicity toward *V. dahliae* microsclerotia. Repeated determination of microsclerotia in naturally infested soils at various localities showed high uniformity. The number of viable microsclerotia recovered from soil per number of microsclerotia introduced × 100 was about 55%.

In recent years, Verticillium wilt has become a serious problem in the Negev region of Israel. Many susceptible field crops and fruit trees have become heavily infected with *Verticillium dahliae* Kleb.–a soil borne pathogen that was introduced to the Negev area via infected potato tubers (12). Owing to the expansion of potato-growing in the Negev, this pathogen multiplied enormously and has become established in many fields in the region.

For practical purposes it has become prerequisite to determine quantitatively the number of microsclerotia in soil. Several methods for the isolation of microsclerotia from soil have been reported. With most techniques, only 20 or more microsclerotia per gram of soil could be detected (5, 7, 9, 11). Lacy and Horner (13) using the first selective medium developed by Nadakavukaren and Horner (14) recovered eight *Verticillium* propagules/gram of soil, but it has been shown that as few as three and one-half microsclerotia per gram soil can cause 100% infection in cotton fields (1). With the most common technique, wet-sieving, the microsclerotia are separated from the soil by differences in size. However from our own experience the fraction which contained the microsclerotia (37-125 μm), still includes soil particles which interferes with the identification of the *Verticillium* colonies. With the wet-sieving technique fewer than one microsclerotium per gram of soil were detected (2).

In neither of the reported isolation techniques were data presented on number of viable microsclerotia recovered from soil per number of microsclerotia introduced. A new quantitative technique for the isolation of small numbers of microsclerotia from soil is described herein; part of the work was reported previously (4).

MATERIALS AND METHODS

Separation of microsclerotia from soil.—Air-dried soil samples of 5 g each were gently ground in a mortar and pestle and shaken in a 250-ml separatory funnel with 20 ml of cesium chloride water solution (1:1, w/v) of 1.6 specific gravity (BDH technical grade) for about 10 seconds. After settling for 45 seconds, the soil precipitate then was removed from the separatory funnel. Cesium chloride solution (8 ml) was pipetted onto the funnel walls to wash down all particles adhering to the glass surface. The mixture was allowed to settle for another 45 seconds, and then all except 2 ml of the cesium chloride soil suspensions was drained. The 2-ml residue which contained the microsclerotia was transferred into a test tube. Fifteen ml of water that was used to rinse the funnel was combined with the 2 ml of residue. The test tube was kept undisturbed for 15 minutes before 12 ml of the solution was decanted. Another 15 ml of water was added to the test tube which was kept undisturbed for 15 minutes. Fifteen ml of the solution was then decanted (the microsclerotia were rinsed twice in order to dilute the cesium chloride and to remove buoyant spores of different fungi). The 2 ml of suspension that contained the microsclerotia was transferred into three petri plates and 10 ml of the medium of Ausher et al. (3) (at 45 C) was poured into each plate. The decanted water (15 ml × 2) was filtered through a Whatman No. 5 filter paper disk (9.0 cm) which then was laid on solidified medium in a petri plate. This last stage was to ensure against possible loss of microsclerotia during the decantation stages. The plates were incubated at 20 C for 24 days and examined under a low-power stereo dissecting microscope to determine the number of Verticillium colonies.

To determine the microsclerotial population in soil, loess soil samples were collected from a potato field in the Negev region of Israel during the autumn of 1974. The samples were air-dried and then kept for another 2 weeks at room temperature before processing, in order to minimize the number of viable *Verticillium* conidia (8).

RESULTS

Effect of cesium chloride solution on germination of

microsclerotia.—Pre-incubation of microsclerotia in cesium chloride solution for 3 hours did not affect their germination rate. No differences could be detected between results of tests with microsclerotia originating from culture media or potato stem debris, or with microsclerotia of different viability (100, 90, 50, and 15%).

Reproducibility.—The variability in microsclerotia numbers in repeated isolation procedures was checked in naturally infested soil. The soil samples were mixed thoroughly to assure maximal homogeneities. High uniformity between replicates at various levels of microsclerotia numbers was obtained (Table 1).

Determination of viable microsclerotia recovered per number of microsclerotia introduced using the cesium chloride flotation technique.—Soil with known amounts of microsclerotia was processed. Zero, 10, 25, and 50 microsclerotia from potato stems were introduced into 5-g soil samples. To avoid the introduction of multiple microsclerotia, each one was examined under a low-power stereo dissecting microscope. Only 48-60% of the total number of microsclerotia could be recovered (Table 2). In the control treatment known numbers of microsclerotia were mixed with soil extracts derived from Verticillium-free soil using the cesium chloride flotation technique. As a result, the germination rate decreased by about 15%, probably due to the interaction between the microsclerotia and other soil microorganisms which were carried over to the agar medium. A further 5% decrease should be attributed to the nonviability of the microsclerotia used. Thus, the cesium chloride flotation technique might enable recovery of 70-80% of the viable microsclerotia.

In another experiment, the number of germinating microsclerotia recovered with the cesium chloride flotation technique was compared with that recovered by the wet-sieving technique. A 100% increase in microsclerotial numbers was obtained with our technique (Table 3).

DISCUSSION

In a search for a suitable medium for the isolation of microsclerotia from soil, three characteristics were sought: (i) a high-specific-gravity solution that would allow flotation of microsclerotia to the surface and allow sedimentation of soil particles to the bottom; (ii) low viscosity, that would assure fast and complete separation of microsclerotia from soil; and (iii) a nonfungitoxic medium. Fifteen substances were screened, among which the cesium chloride solution proved most suitable for our purpose. Other substances that possess high specific gravity were either too viscous or fungitoxic toward microsclerotia of *V. dahliae*.

Huisman and Ashworth (10) recently reported the use of sucrose solution for the isolation of Verticillium microsclerotia from soil. They found this method to be less efficient and more variable than the wet-sieving technique. Owing to its high viscosity, sucrose solution was found to be less adequate than cesium chloride for our assay. With the wet-sieving technique, 10-20% of the microsclerotia usually escaped through the 37-μm sieve (10); another 10-14% were retained on the 125-μm sieve

TABLE 2. Recovery of viable microsclerotia of *Verticillium dahliae* from soil with the cesium chloride flotation technique[a]

Number of microsclerotia/5 g soil	Percentage germination of microsclerotia in agar medium	Percentage recovery[b]	
		Control[c]	Soil
0	. . .	0	0
10	95	80	60
25	95	80	56
50	95	80	48

[a]Each number represents a mean of three replicates with the control treatments and of five replicates with the soil treatment.

[b]Number of colonies detected per number of microsclerotia introduced × 100.

[c]The microsclerotia were combined with soil extract (derived by the cesium chloride flotation technique) before being mixed with the selective agar medium.

TABLE 3. Comparative determination with the cesium chloride flotation and wet-sieving techniques of numbers of *Verticillium dahliae* microsclerotia in a naturally infested soil.

Replicate no.	Number of microsclerotia/5 g soil	
	Cesium chloride flotation technique	Wet-sieving technique
1	32	18
2	32	19
3	39	22
4	33	13
5	40	14
Mean	35.5	17.5

TABLE 1. Reproducibility of recovery of viable microsclerotia of *Verticillium dahliae* in various naturally infested soils by the cesium chloride flotation technique

Replicate no.	Number of microsclerotia recovered/5 g Soil sample no.:														
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV
1	99	39	32	26	22	13	11	9	6	4	9	4	4	1	0
2	108	34	32	23	17	8	7	6	9	4	7	6	4	1	0
3	113	35	39	21	16	9	12	8	6	7	6	4	6	1	1
4	121	38	33	29	17	8	9	8	6	5	8	6	4	2	0
5		35	40	23	18	8	7	8	7	4	6	4	4	1	1
6		30	37				10								
Mean	109	35	35	24	18	9	9	8	7	5	7	5	4	1	0.4

(Ben-Yephet and Pinkas, *unpublished*). Thus, 20-34% of the total microsclerotia present in the sample were undetectable with this technique. With most techniques (5, 7, 9, 11) fewer than 20 microsclerotia per gram of soil can not be detected. It was found that 1-2 microsclerotia per gram of soil can cause 100% incidence of infected watermelon plants grown under plastic cover during the winter (Krikun and Ben-Yephet, *unpublished*). A quantitative root assay was recently described by Evans et al. (6), who found a positive linear correlation between the number of *Verticillium dahliae* colonies developing on 100 cm of plant root and the number of microsclerotia in the soil. It is still questionable whether fewer than 10 microsclerotia per gram of soil can be determined accurately with that technique.

The cesium chloride flotation technique enabled detection of about 55% of the total microsclerotia present in soil (Table 2), with low variability between replicate samples (Table 1). This technique enables the concentration of microsclerotia in the soil (about 100-fold) and thus facilitates detection of lower numbers of Verticillium colonies in the soil. In a comparative experiment, twice as many microsclerotia were detected with our technique as with the wet-sieving technique. We found it possible to re-use the cesium chloride; thus, 40-50 soil samples of 5 g each required only 100 g of cesium chloride salt. This method was found to be very useful in the loess soil of the Negev region, in which small numbers of microsclerotia have been found to cause a high rate of plant infection.

LITERATURE CITED

1. ASHWORTH, L. J., JR., O. D. MC CUTCHEON, and A. G. GEORGE. 1972. Verticillium albo-atrum: the quantitative relationship between inoculum density and infection of cotton. Phytopathology 62:901-903.
2. ASHWORTH, L. J., JR., J. E. WATERS, A. G. GEORGE, and O. D. MC CUTCHEON. 1972. Assessment of microsclerotia of Verticillium albo-atrum in field soils. Phytopathology 62:715-719.
3. AUSHER, R., J. KATAN, and S. OVADIA. 1975. An improved selective medium for the isolation of Verticillium dahliae. Phytoparasitica 3(2):133-137.
4. BEN-YEPHET, Y., and Y. PINKAS. 1975. Quantitative isolation of Verticillium dahliae from soil. Phytoparasitica 3:70. (Abstr.).
5. DE VAY, J. E., and L. L. FORRESTER. 1972. Nature and concentration of propagules of Verticillium dahliae in field soils relative to the prevalence of Verticillium wilt in cotton. Phytopathology 62:754 (Abstr.).
6. EVANS, G., C. D. MC KEEN, and A. C. GLEESON. 1974. A quantitative bioassay for determining low numbers of microsclerotia of Verticillium dahliae in field soils. Can. J. Microbiol. 20:119-124.
7. EVANS, G., S. WILHELM, and W. C. SNYDER. 1967. Quantitative studies by plate counts of propagules of the Verticillium wilt fungus in cotton field soils. Phytopathology 57:1250-1255.
8. GREEN, R. J., JR., 1969. Survival and inoculum potential of conidia and microsclerotia of Verticillium albo-atrum in soil. Phytopathology 59:874-876.
9. HARRISON, M. D., and C. H. LIVINGSTON. 1966. A method for isolating Verticillium from field soil. Plant Dis. Rep. 50:897-899.
10. HUISMAN, O. C., and L. J. ASHWORTH, JR. 1974. Verticillium albo-atrum: Quantitative isolation of microsclerotia from field soils. Phytopathology 64:1159-1163.
11. ISAAC, I., P. FLETCHER, and J. A. C. HARRISON. 1971. Quantitative isolation of Verticillium spp. from soil and moribund potato haulm. Ann. Appl. Biol. 67:177-183.
12. KRIKUN, J., D. NETZER, and N. SOFER. 1974. The role of soil fumigation under condition of intensive agriculture. Agro-Ecosystems 1:117-122.
13. LACY, M. L., and C. E. HORNER. 1965. Verticillium wilt of mint: Interaction of inoculum density and host resistance. Phytopathology 55:1176-1178.
14. NADAKAVUKAREN, M. J., and C. E. HORNER. 1959. An alcohol agar medium selective for determining Verticillium microsclerotia in soil. Phytopathology 49:527-528.